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(54) Title: B70(B7-2):CTLA-4 BONDING PROTEIN

(57) Abstract

B70 antigen from a mammal, and reagents relating thereto, including purified nucleic acids encoding such proteins, proteins, and specific antibodies, are provided, together with methods of using said reagents and diagnostic kits using said reagents.

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B70(B7-2):CTLA-4 BONDING PROTEIN

FIELD OF THE INVENTION

The present invention pertains to compositions related to proteins which function in controlling physiology, development, and/or differentiation of mammalian cells, e.g., cells of a mammalian immune system. In particular, it provides proteins and mimetics which regulate physiology, development, differentiation, and/or function of various cell types, including hematopoietic cells. These reagents are related to a heretofore unidentified cell marker which interacts with CTLA-4 and CD28 cell markers.

BACKGROUND OF THE INVENTION

T lymphocytes recognize antigen via the CD3/T cell antigen receptor (TcR) complex. Binding of anti-CD3 or anti-TcR monoclonal antibodies (mAb) to T cells results in a rapid increase in intracellular Ca^{++} and the generation of inositol triphosphate (IP_3): see Imboden et al., (1985) J. Exp. Med. 161:446-456; Weiss et al., (1984) Proc. Natl. Acad. Sci. USA 81:4169-4173; Weiss et al., (1984) Proc. Natl. Acad. Sci. USA 81:6836-6840; and Weiss et al., (1986) Ann. Rev. Immunol. 6:593-619. Although cross-linking of CD3/TcR alone is often sufficient to induce inositolphosphate pathway activation, other signals are apparently necessary to induce functions such as cytokine secretion and proliferation. Soluble factors and interaction with cell surface receptors have both been implicated as co-stimulators of CD3/TcR-mediated activation. Thus, antigen-specific recognition alone may be insufficient to trigger effector functions since additional accessory molecules are required for an efficient response.

CD28 is a disulfide-linked homodimer that is expressed on the majority of human peripheral blood T cells, including T lymphocytes and thymocytes: see Hansen et al., (1980) Immunogenetics 10:247-260; Hara et al., (1985) J. Exp. Med. 161:1513-1524; and Linsley et al., (1993) Ann. Rev. Immunol. 11:191-212. Monoclonal antibodies against CD28 in conjunction with phorbol ester induce T cell proliferation and augment proliferation induced by anti-CD3 or anti-CD2 mAb: see Martin et al., (1986) J. Immunol. 136:3282-3287; Yang et al., (1988) J. Exp. Med. 168:1457-1468; Damle et al., (1988) J. Immunol. 140:1753-1524; van Lier et al., (1988) Eur. J. Immunol. 18:167-172; and Pierres et al., (1988)

Eur. J. Immunol. 18:685-690. Anti-CD28 mAb-induced proliferation possibly results from stabilization of messenger RNA for IL-2 and other cytokines.

Recently, it has been demonstrated that a B cell activation antigen, B7 (Freedman et al., (1987) J. Immunol. 139:3260-3267) or BB1 (Yokochi et al., (1982) J. Immunol. 128:823-827), is a natural binding partner for CD28 and that a specific binding interaction mediates heterotypic adhesion (Linsley et al., (1991) J. Exp. Med. 173:721-730). Interaction of CD28 and B7 results in augmentation of T cell proliferation and cytokine production, and antibodies against CD28 or B7 inhibit alloantigen and mitogen-induced proliferative responses: see Koulova et al., (1991) J. Exp. Med. 173:759-762. B7 is expressed at low levels on resting B cells and monocytes, and is elevated following stimulation with pokeweed mitogen, anti-Ig, anti-HLA class II, or Epstein Barr Virus (EBV). Therefore, it is likely that T cell activation via CD28 depends on the capacity of the antigen-presenting cells to up-regulate expression of B7. CD28/B7 interactions are important in the generation of human cytotoxic T lymphocytes and in the activation of T cells against alloantigens, an event important in transplantation rejection: see Azuma et al., (1992) J. Exp. Med. 175:353-360; Azuma et al., (1993) J. Immunol. 177:2091-2101; and Paul (ed.) (1989) Fundamental Immunology Raven Press, New York, NY.

B7 glycoprotein binds to another membrane antigen, CTLA-4, in addition to CD28: see Linsley et al., (1991) J. Exp. Med. 174:561-569. CTLA-4 is expressed on activated T lymphocytes and is structurally very similar to CD28: see Brunet et al., (1987) Nature 328:267-270; Brunet et al., (1988) Immunol. Rev. 103:21-36; Dariavach et al., (1988) Eur. J. Immunol. 18:1901-1905; Harper et al., (1991) J. Immunol. 147:1037-1044; and Balzano et al., (1992) Int. J. Cancer Supplement 7:28-32. Partner binding to CTLA-4 seems important also in T cell activation: see Linsley et al., (1992) J. Exp. Med. 176:1595-1604.

Whereas B7 is an important binding partner for CTLA-4 and CD28, other specific partners which bind to CTLA-4 and CD28 may exist, and their properties and biological effects will be important for both diagnostic and therapeutic uses. In particular, alternative agonists and antagonists of the B7/BB1 proteins will often serve as substitutes under appropriate circumstances and will often have differences which can be exploited to therapeutic advantage. These differences may reflect specificity of binding, affinity, or other important properties. Moreover, signal transduction may be controllable using combinations of agonists or antagonists for the different binding partners for CTLA-4/CD28.

The absence of complete knowledge about the structural, biological, and physiological properties of partners which naturally bind to CTLA-4/CD28

prevents full modulation of the effects of these markers. Various medical conditions, e.g., neoplastic proliferation, graft vs. host reactions, and autoimmune disorders, reflect inappropriate regulation of the development or physiology of relevant cells. Thus, many abnormal immune responses remain unmanageable. However, the present invention provides solutions to many problems of these types.

SUMMARY OF THE INVENTION

The present invention is based, in part, upon the discovery of a natural proteinaceous binding partner for CTLA-4 and CD28 markers/antigens. This binding partner is distinct from the known B7/BB1 marker which binds to CTLA-4 and CD28. This new partner will be referred to as B70, and is representative of here designated "type B" markers/antigens/binding partners, typically cell surface proteins/antigens, which were initially characterized by their ability to bind specifically to "type A" markers/antigens/binding partners. The type A markers/binding partners include both CTLA-4 and CD28, and antibodies specific for B70, and are characterized, in part, by their property of serving as a binding partner for B70; see Table 1.

Table 1. Type A and type B binding counterparts.

type A examples:	type B examples:
CTLA-4	B7/BB1
CD28	B70
antibodies to B7/BB1	antibodies to CTLA-4
antibodies to B70	antibodies to CD28

The type B antigens/binding partners are exemplified by B70, and either are binding partners to type A markers/antigens, or are structurally or functionally related to B70, and include biological variants of B70. The known B7/BB1 antigens are distinct from the novel B70 compounds are therefore are not included in the definition of the patent claims directed to binding partners. Functionally, the type A and type B markers/antigens are binding partners for one another, and the specific binding interaction can lead to a physiological response in a cell possessing one or the other marker.

The invention embraces agonists and antagonists of this natural B70 protein, e.g., mutants (muteins) of the natural sequence, fusion proteins,

chemical mimetics, certain antibodies, and other structural or functional analogs; but all distinct from known B7/BB1. It is also directed to isolated genes encoding proteins of the invention. Various uses of these different nucleic acid or protein compositions are also provided.

The present invention provides a substantially pure nucleic acid homologous to a sequence encoding a B70 protein or fragment thereof; a B70 protein or peptide thereof, or a fusion protein comprising B70 protein sequence; and an antibody specific for binding to a B70 protein made by immunizing with purified B70.

In nucleic acid embodiments, the nucleic acid can comprise a nucleic acid from a warm-blooded animal selected from the group of birds and mammals, including a rodent or primate; a nucleic acid exhibiting substantial nucleotide homology to at least one nucleotide segment of SEQ ID NO: 1; or a nucleic acid encoding a B70 protein.

In embodiments relating to substantially pure B70 protein or to peptides thereof, the protein or peptide can be from a warm-blooded animal selected from the group of birds and mammals, including a rodent or primate, e.g., human; comprise at least one polypeptide segment of SEQ ID NO: 2; exhibit a post-translational modification pattern distinct from natural B70 protein; or exhibit at least one of the activities characteristic of a natural B70 protein, e.g., binding to CTLA-4 or CD28 and conferring resulting biological responses. A further embodiment is a composition comprising such a binding partner (type B) and either a pharmaceutically acceptable carrier or another biologically active ingredient.

In antibody embodiments, the antibody will often be raised against a peptide sequence of SEQ ID NO: 2; the antibody is a monoclonal antibody; or the antibody is labeled. The antigen recognized by the antibody can be a mammalian protein including a rodent or primate, e.g., human.

The invention also embraces a kit comprising a substantially pure nucleic acid which encodes, or is complementary to one which encodes a B70 protein or peptide, e.g., as a diagnostic positive control; a B70 protein or fragment; or an antibody which is raised against a B70 protein.

The availability of these reagents also provides methods of modulating physiology, growth, or development of a cell comprising contacting or exposing said cell with an agonist or antagonist of a B70 protein. For example, the antagonist might be an antibody raised against a mammalian B70 protein or the cell may be a hematopoietic cell, including a lymphoid cell; or non-lymphoid cells.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show a FACS analysis of COS7 cells transfected with B70 cDNA plasmid LL278, an expression vector expressing the B70 antigen; Figure 1C is a control (mock-transfection). Cells were stained with IT2.236 (anti-B70) monoclonal antibody (shown by X in Figure 1A), with CTLA-4-Ig fusion (shown by Y in Figure 1B), and with IT2.236 (shown by Z in Figure 1C); in all these Figures, Q indicates a control.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

GENERAL

The present invention is based, in part, upon the discovery of a natural binding partner, e.g., type B binding compositions, which can bind to and activate a cell via the CTLA-4 and/or CD28 marker/antigens. These proteins will be referred to as B70 markers/antigens and are distinct from the known B7/BB1 marker which binds to CTLA-4 and CD28. The B70 antigen described herein is, in its full length natural embodiment, a 70 kD glycoprotein derived from humans and composed of a core protein of about 40 kD, with N-linked oligosaccharides. This protein is found on various cell types involved in the immune system, e.g., antigen-presenting cells (which include monocytes, activated B cells, dendritic cells, and activated T cells). The full length B70 can activate cells through binding to CD28 or CTLA-4 markers/antigens, e.g., type A binding compositions.

It has been observed that anti-human B7 antibodies, e.g., mAb L307, could completely block the binding of CTLA-4-Ig fusion protein to murine L cells, murine P815 mastocytoma cells, or human Jurkat T leukemia cells stably transfected with human B7 cDNA. However, Applicants have now recognized that these antibodies only partially blocked binding of CTLA-4-Ig to EBV-transformed human B lymphoblastoid cell lines, e.g., JY cells, activated human T cell clones, dendritic cells, and activated NK cell clones. This suggested that other binding partners, e.g., B70, exist for CTLA-4 and are expressed in the B lymphoblastoid cells, activated NK cells, and activated T cells.

In addition, it was observed that a human NK leukemia cell line YT (a cell expressing CD28, but not CTLA-4) killed cells expressing B7 and the above cell lines transfected with human B7 cDNA. Killing of those cell lines was efficiently blocked by anti-CD28 mAb, thus implicating a CD28-dependent interaction: see

Azuma et al., (1992) J. Immunol. 149:1115-1123. Of particular importance, Applicants have now observed that killing of the B7 transfectants was efficiently blocked by anti-B7 mAb, whereas killing of the EBV transformed B lympho-blastoid cell lines, e.g. JY and others, was only partially blocked by anti-B7. Since YT expresses CD28 but lacks CTLA-4, this suggested the existence of other binding partners for CD28 and therefore the existence of novel binding partners for CTLA-4 or CD28 other than B7/BB1. Moreover, it is likely that this novel binding partner for CTLA-4 or CD28 is important in immune responses, including transplantation rejection and tumor immunity.

The present invention provides the nucleotide sequence encoding portions of various mammalian proteins designated B70 or type B proteins, which are characterized as binding partners for CTLA-4 or CD28 markers, but are distinct from the known B7/BB1. The natural B70 proteins are capable of mediating various biochemical responses which should lead to biological or physiological responses in cells bearing CD28 or CTLA-4. Biological activities of B70 will typically overlap with those of B7/BB1. The best characterized embodiment of this invention is described in primates, e.g., humans. Similar sequences for proteins in other mammalian species, e.g., in the mouse, are also made available by the present disclosure. The descriptions below are directed, for exemplary purposes, to a human B70 protein, but are likewise applicable to related embodiments from other animal species.

The CTLA-4 and CD28 markers, which each exhibit properties of a binding partner to B7, mediate a biochemical response to binding of a heretofore unidentified protein, leading to signal transduction and cellular response. In particular, the protein has been isolated by use of a monoclonal antibody IT2.1 (originally designated IT2.236.20) which blocks or neutralizes non-B7/BB1 mediated effects via CTLA-4 and CD28: see Aruffo and Seed (1987) Proc. Nat'l Acad. Sci. USA 84:8573-8577. Antibody binding has identified a cell which expresses a protein whose interaction with the CTLA-4 and CD28 markers can be blocked using the neutralizing antibody.

B70 should be expressed in antigen-presenting cells, e.g., dendritic cells, monocytes, and activated B cells, and the interaction of B70 with interacting cells should be important for mediating various aspects of cellular physiology or development. In particular, B70 presentation should augment immune T-cell responses and stimulate T-cell-mediated immune responses, e.g., for treating immunosuppressed or immunocompromised patients, as a supplement in an immunization protocol, or as a stimulator of immune surveillance in tumor therapy. B70 antagonists would block some responses, e.g., those due to

hypersensitivity, including allergic responses, or to transplantation. These may be valuable in in vitro or in vivo contexts. Identification of a binding partner for CTLA-4 and CD28 but distinct from B7/BB1 provides means to address some of the questions raised by these observations.

Nucleic Acids

The described isolated cDNA clone and nucleotide sequences are useful for isolating homologous genes or for expressing a type B binding partner, e.g., B70 protein or fragments thereof. Further characterization of the protein also will simplify purification of the proteins, e.g., from a natural or recombinant protein expression source. Typically, the isolated nucleic acid clones will be useful in isolating a homologous gene from other species, e.g., warm-blooded animals, such as birds and mammals. Cross hybridization will allow isolation of variants, e.g., allelic variants or variants from other species. A number of different approaches should be available to isolate suitable nucleic acid clones successfully using the reagents made available herein. Many standard techniques of molecular biology are available to isolate larger clones, e.g. full length clones, or homologous sequences from various sources. Isolation of flanking sequences is also easily performed: see, e.g., Sambrook et al. and Ausubel et al.

The nucleotide segments and complementary sequences can also be used to screen a library. Appropriate oligonucleotide primers will be useful as probes for screening by polymerase chain reaction (PCR) methods, or as probes for hybridization: see, e.g., SEQ ID NO: 1. In combination with PCR techniques, synthetic oligonucleotides will be useful in selecting correct clones from a library. Complementary sequences will also be used as probes, primers, or as antisense reagents. The entire coding segment of one isolate of the B70 is disclosed in SEQ ID NO: 1.

This invention contemplates use of isolated DNA or fragments to encode a biologically active type B protein, e.g., a B70 protein or polypeptide. In addition, this invention covers isolated or recombinant DNA which encodes a biologically active protein or polypeptide and is capable of hybridizing under appropriate, e.g., stringent, conditions with the DNA sequences described herein. Said biologically active protein or polypeptide can be an intact ligand, or fragment, and have an amino acid sequence as disclosed in SEQ ID NO: 2. Further, this invention covers the use of isolated or recombinant DNA, or fragments thereof, which encode proteins which are homologous to a B70 protein or

which are isolated using cDNA encoding a B70 protein as a probe. The isolated DNA can have the respective regulatory sequences in the 5'- and 3'-flanking regions, e.g., promoters, enhancers, poly-A addition signals, and others.

An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other components which naturally accompany a native sequence, e.g., ribosomes, polymerases, and distantly flanking, e.g., 5-50 kilobases, genomic sequences from the originating species. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and typically encompasses recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule.

An isolated nucleic acid will generally be a homogeneous composition of molecules, but will, in some embodiments, possess some heterogeneity. This heterogeneity will typically be found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is defined either by its method of production or by its structure. In reference to its method of production, e.g., a process for making a product, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence, typically selection or production, and generally using some *in vitro* steps. In reference to its structure, it can be a nucleic acid consisting of two fused fragments which are not naturally contiguous to each other, but it is meant to exclude products of nature, e.g., naturally occurring mutants. Thus, for example, unnaturally occurring vectors made for transforming cells are encompassed, as are nucleic acids comprising sequences derived using any synthetic oligonucleotide process. Such a process is often used to replace a codon with a redundant codon encoding the same amino acid or a conservative amino acid substitution, while typically introducing or removing a sequence-recognition site.

Alternatively, this is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site-specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features, may be incorporated by design. (A similar concept also applies to a recombinant polypeptide, e.g., a fusion polypeptide.) Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode polypeptides similar

to fragments of these antigens, and fusions of sequences from various different molecular variants.

A significant "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 23 or 29 nucleotides, often at least 35 or 41 nucleotides, preferably at least 47 or 53 nucleotides, and in particularly preferred embodiments will be of 56 or more nucleotides.

A DNA which encodes a B70 protein will be particularly useful to identify genes, mRNA, and cDNA variants which code for related or homologous proteins, as well as DNAs which code for homologous proteins from different species. Probably there are homologues in other species, including rodents or primates other than humans. Various B70 proteins should be homologous and are encompassed herein. However, even DNA encoding proteins that have a more distant evolutionary relationship to the protein can readily be isolated under lower stringency conditions using these sequences if they are sufficiently homologous. Primate B70 proteins and encoding DNA are of particular interest.

This invention further covers recombinant DNA molecules and fragments having a DNA sequence identical to or highly homologous to the isolated DNAs set forth herein. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. Alternatively, recombinant clones derived from the genomic sequences, e.g., containing introns, will be useful for transgenic studies, including, e.g., transgenic cells and organisms, and for gene therapy: see, e.g., Goodnow (1992) "Transgenic Animals" in Roitt (ed.) Encyclopedia of Immunology Academic Press, San Diego, pp. 1502-1504; Travis (1992) Science 256:1392-1394; Kuhn et al., (1991) Science 254:707-710; Capecchi (1989) Science 244:1288; Robertson (1987) (ed.) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, IRL Press, Oxford; and Rosenberg (1992) J. Clinical Oncology 10:180-199.

Homologous nucleic acid sequences, when compared, exhibit significant similarity. The standards for homology in nucleic acids either are measures for homology generally used in the art by sequence comparison or are based upon hybridization conditions. The hybridization conditions are described in greater detail below.

Substantial homology in the context of comparing nucleic acid sequences means that the segments are identical when optimally aligned, allowing for appropriate nucleotide insertions or deletions, in at least about 50% or even 58% of the nucleotides, generally at least 65% or even 71%, usually at least about 77% or even 85%, preferably at least about 95 to 98% or more, and, in

particular embodiments, as many as about 99% or more of the nucleotides. The comparison can also be made with a complementary strand on the basis of the specific nucleotide pairing that dsDNA uses.

Alternatively, substantial homology exists when the segments will hybridize under highly selective hybridization conditions to a strand or to its complement, typically using a sequence derived from SEQ ID NO: 1. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%: see Kanehisa (1984) Nuc. Acids Res. 12:203-213. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 24 nucleotides, typically at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides.

"Stringent conditions", in referring to hybridization in the context of homology, will be stringent combined conditions of salt, temperature, organic solvents, and other variables, typically those controlled in hybridization reactions. Stringent temperature conditions will usually include temperatures in excess of about 30°C, typically in excess of about 45°C, preferably in excess of about 65°C, and more preferably in excess of about 70°C. Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, typically less than about 300 mM, preferably less than about 200 mM, and more preferably less than about 150 mM. However, the combination of variables is much more important than the measure of any single variable: see, e.g., Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370.

The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A commercial oligonucleotide synthesizer may be used, e.g., Applied Biosystems 392 and 394 synthesizer machines. See also operation manuals which accompany the machines. A double-stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using a DNA polymerase with an appropriate primer sequence. This allows means for generating modified sequences from a natural sequence at specific sites.

B70 from other mammalian species can be cloned and isolated by cross-species hybridization of closely related species. Homology may be relatively low between distantly related species, and thus hybridization of relatively closely

related species is advisable. Alternatively, preparation of an antibody preparation which exhibits less species specificity may be useful in expression cloning approaches.

Purified B70 protein

Sequences of fragments of the human B70 protein sequence are shown in SEQ ID NO: 2. These amino acid sequences, provided amino to carboxy, are important in providing partial sequence information from the type B binding partner thereby allowing for distinguishing the protein from other proteins. Moreover, the peptide sequences allow preparation of peptides to generate antibodies to recognize such segments, and allow preparation of oligonucleotide probes, both of which are strategies for isolation, e.g., cloning, of genes encoding such sequences.

As used herein, the term "human B70" shall encompass, when used in a protein context, a protein containing amino acid sequences shown in SEQ ID NO: 2, or a significant fragment of such a protein. It also refers to a human derived polypeptide, distinct from the known B7/BB1, which exhibits similar biological function as B70, or interacts with B70-specific binding components such as the type A binding partners CTLA-4 and CD28. These type A proteins, including antibodies recognizing B70, will often bind to a B70 protein with high affinity, e.g., at better than about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM. Homologous B70 proteins would be found in mammalian species other than human, e.g., rats and mice. Non-mammalian species should also possess structurally or functionally related genes and proteins.

The term "polypeptide" as used herein includes a significant fragment or segment, and encompasses a stretch of amino acid residues of at least about 8 amino acids, generally at least 12 or 16 amino acids, preferably at least 20 or 24 amino acids, and, in particularly preferred embodiments, at least 28 or even 30 or more amino acids.

The term "type A binding composition" refers to molecules that bind with specificity to B70, e.g., in an antibody-antigen interaction, or compounds, e.g., proteins which specifically associate with B70, e.g., in a natural physiologically relevant protein-protein interaction, either covalent or non-covalent. The interaction between B70 and CTLA-4/CD28 can be characterized as interaction of binding partners, but is also described as a type A (e.g., CTLA-4/CD28) to type B (e.g., B70) binding-partner interaction. The binding compositions may be

a polymer (e.g., proteinaceous), or chemical reagent. A functional analog may be a protein with structural modifications, or may be a wholly unrelated molecule, e.g., which has a molecular shape which interacts with the appropriate marker binding determinants. B70 homologues distinct from B7/BB1 may serve as agonists or antagonists for type A molecules CTLA-4 or CD28: see, e.g., Goodman et al., (eds.) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed.), Pergamon Press. Antibodies, the CTLA-4 and CD28 markers, and soluble binding versions are preferred compositions which specifically bind B70, e.g., type B binding partners.

Substantially pure, in a protein context, typically means that the protein is free from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, generally at least about 60% pure, often at least about 75% pure, typically at least about 85% pure, preferably at least about 95% pure, and in most preferred embodiments, at least about 99% pure.

Solubility of a polypeptide or fragment depends upon the environment and the polypeptide. Many variables affect polypeptide solubility, including temperature, electrolyte environment, size and molecular characteristics of the polypeptide, and nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4°C to about 65°C. Usually the temperature at use is greater than about 18°C and more usually greater than about 22°C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually be body temperature, typically about 37°C for humans, though under certain situations the temperature may be raised or lowered in situ or in vitro.

The electrolytes will usually approximate in situ physiological conditions, but may be modified to higher or lower ionic strength where advantageous. The actual ions may be modified, e.g., to conform to standard buffers used in physiological or analytical contexts.

The polypeptide should generally be in a substantially stable state, and usually not in a denatured state. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility, or associated with lipids or detergents, e.g., in a manner which approximates natural lipid bilayer interactions. In particular embodiments, constructs of proteins that normally lodge in cell membranes can often be made soluble by truncating an

expression product to delete hydrophobic regions, e.g., membrane-spanning or membrane-associated protein domains.

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. Usually the solvent will have a neutral pH, typically between about 5 and 10, and preferably about 7.5. On some occasions, a detergent will be added, typically a mild non-denaturing one, e.g., CHS (cholestanyl hemisuccinate) or CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), in a low enough concentration to avoid significant disruption of structural or physiological properties of the ligand.

Solubility is often reflected by sedimentation measured in Svedberg units, which is a measure of the sedimentation velocity of a molecule under particular conditions. The determination of the sedimentation velocity was classically performed in an analytical ultracentrifuge, but is typically now performed in a standard ultracentrifuge: see Freifelder (1982) Physical Biochemistry (2d ed.), W.H. Freeman; and Cantor and Schimmel (1980) Biophysical Chemistry, parts 1-3, W.H. Freeman & Co., San Francisco. As a crude determination, a sample containing a putatively soluble polypeptide is spun in a standard full-sized ultracentrifuge at about 50,000 rpm for about 10 minutes, and soluble molecules will remain in the supernatant. A soluble particle or polypeptide will typically be less than about 30S, usually less than about 10S, and, in particular embodiments, less than about 4S, and preferably less than about 3S.

Physical Variants

This invention also encompasses proteins or peptides whose amino acid sequence shows substantial homology with the amino acid sequence of B70. The variants include species, allelic, and metabolic variants.

Homology of two (or more) amino acid sequences, or degree of sequence identity, is determined by optimizing residue matches, if necessary by introducing gaps as required. This changes when substitutions with conservative amino acid residues are regarded as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are typically intended to include natural allelic and interspecies variations in each respective protein sequence.

Typical homologous proteins or peptides will have from 25-100% homology (if gaps can be introduced), to 50-100% homology (if conservative substitutions are included) with the amino acid sequence of the B70 protein. Homology measures will be at least about 35%, generally at least about 40% or 50%, often at least about 60% or 70%, preferably at least about 80%, and in particularly preferred embodiments, at least about 85% or more: see also Needleham et al., (1970) J. Mol. Biol. 48:443-453; Sankoff et al., (1983) Chapter One in Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group, Madison, WI.

The isolated B70 protein DNA can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode these antigens, their derivatives, or proteins having similar physiological, immunogenic, or antigenic activity. These modified sequences can be used to produce mutant antigens or to enhance expression. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant B70 protein derivatives include predetermined or site-specific mutations of the respective protein or its fragments. "Mutant B70" encompasses a polypeptide otherwise falling within the homology definition of the human B70 as set forth above, but having an amino acid sequence which differs from that of B70 protein as found in nature, whether by way of deletion, substitution, or insertion. In particular, the term "site-specific mutant B70 protein" generally includes proteins having significant homology with a protein containing sequences of SEQ ID NO: 2, and sharing various biological activities, e.g., antigenic or immunogenic, with those sequences, and in preferred embodiments contains most of the disclosed sequences and/or a full-length natural protein. Similar concepts apply to different B70 proteins, particularly those found in various warm-blooded animals, e.g., mammals and birds. As stated before, it is emphasized that descriptions are generally meant to encompass all B70 proteins, not limited to the human embodiment specifically discussed herein.

Although site-specific mutation sites are predetermined, mutants need not be site-specific. B70 protein mutagenesis can be conducted by making amino acid insertions or deletions. Substitutions, deletions, insertions, or any combinations of these may be generated to arrive at a final construct. Insertions include amino- or carboxy-terminal fusions. Random mutagenesis can be

conducted at a target codon and the expressed mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis or PCR techniques: see also Sambrook et al., (1989) and Ausubel et al., (1987 and Supplements).

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from these proteins. A heterologous fusion protein is a fusion of proteins or segments which in nature are not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a B70 protein polypeptide is a continuous protein molecule having sequences fused, e.g., in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional domains from other proteins. For example, partner-binding segments or other segments may be exchanged between different new fusion polypeptides or fragments: see, e.g., Cunningham et al., (1989) Science 243:1330-1336; and O'Dowd et al., (1988) J. Biol. Chem. 263:15985-15992. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of marker-binding specificities and other functional domains.

The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double-stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence, e.g., primer extension techniques.

Functional Variants

The blocking of physiological response to B70 proteins may result from the inhibition of binding of the type B proteins to their type A binding partners, e.g., CTLA-4/CD28, probably through competitive inhibition. Thus, in vitro assays of the present invention will often use isolated protein, membranes from

cells expressing a recombinant B70 protein, soluble fragments comprising partner-binding segments of these proteins, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of mutations and modifications of either binding segment, or of B70 itself, e.g., of B70 analogs.

This invention also contemplates the use of competitive drug-screening assays, e.g., where type A compositions or neutralizing antibodies to B70 compete with a test compound for binding to a type B composition, e.g., B70. In this manner, the type A compositions can be used to detect the presence of any polypeptide which shares one or more antigenic binding sites of B70, and can also be used to occupy binding sites on the B70 that might otherwise interact with type A proteins, e.g., CTLA-4/CD28.

Additionally, neutralizing antibodies against the B70 protein or soluble fragments of type A binding partners for B70 can be used to inhibit B70 action on particular cell types, thereby affecting B70 action on various tissues, e.g., tissues experiencing abnormal physiology, either in vitro or in vivo.

"Derivatives" of the B70 protein include amino acid sequence mutants, glycosylation variants, and covalent conjugates or aggregate conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in the B70 amino acid side chains or at the N- or C-termini, by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C2 to C18 normal alkyl.

In particular, glycosylation alterations are included, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further processing steps. A particularly preferred means for accomplishing this is exposing the polypeptide to carbohydrate-metabolizing enzymes derived from cells that normally provide such processing, e.g., mammalian glycosylation or deglycosylation enzymes. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

A major group of derivatives comprises covalent conjugates of the B70 protein or fragments thereof with other proteins or polypeptides. Such

derivatives can be synthesized in recombinant culture as N- or C-terminal fusions. Alternatively, they can be formed by coupling the B70 protein or fragments thereof with agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred sites for forming derivatives of the protein with cross-linking agents are at free amino groups, carbohydrate moieties, and cysteine residues.

Fusion polypeptides between the B70 proteins and other homologous or heterologous proteins are also provided. Many growth factors and cytokines are homodimeric entities, and a dimeric construct may have various advantages, including lessened susceptibility to proteolytic cleavage. Homologous polypeptides may be fusions between different surface markers, resulting in, e.g., a hybrid protein exhibiting CTLA-4-binding specificity. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a CTLA-4-binding segment or domain, so that the presence or location of the fusion protein may be easily determined: see, e.g., Dull et al., U.S. Patent No. 4,859,609. Other gene fusion partners include bacterial β-galactosidase, trpE, Protein A, β-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor: see, e.g., Godowski et al., (1988) Science 241:812-816.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands, His 6, or FLAG.

Fusion proteins will typically be made by either recombinant nucleic acid methods or synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for example, in Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232: 341-347; and Atherton et al., (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford.

This invention also contemplates the use of derivatives of the B70 proteins other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall into the three classes: (1) salts, (2)

side-chain and terminal-residue covalent modifications, and (3) adsorption complexes, e.g., with cell membranes. Such covalent or aggregative derivatives are useful for example as immunogens, as reagents in immunoassays, or in purification methods, e.g., in affinity purification of proteins. For example, a B70 antigen can be immobilized by covalent bonding to a solid support such as cyanogenbromide-activated SEPHAROSE, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of anti-B70 antibodies or a binding partner for B70. The B70 proteins can also be labeled with a detectable group, e.g., radio-iodinated by the chloramine-T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic assays. Purification of B70 protein may be effected by immobilized antibodies or binding partners, e.g., type A antigens. Certain derivatives may also exhibit desired storage or stability properties.

The purified protein or defined peptides are useful for generating antibodies by standard methods, as described below. Synthetic peptides or purified protein can be presented to an immune system to generate monoclonal or polyclonal antibodies: see, e.g., Coligan (1991 and periodic supplements) Current Protocols in Immunology Wiley/Greene; and Harlow and Lane (1989) Antibodies: A Laboratory Manual Cold Spring Harbor Press. Alternatively, the CTLA-4 or CD28 or soluble binding equivalents, e.g., type A reagents, can be used as specific binding counterparts for type B proteins, and advantage can be taken of their specificity of binding, much as an antibody would be used.

For example, the type A specific binding composition could be used for screening of an expression library made from a cell line which expresses a B70 protein. The screening can include standard staining of surface expressed type B protein, or panning. Screening of intracellular expression can also be performed by various staining or immunofluorescence procedures. Type A binding compositions could be used for affinity purification or to sort out cells expressing the type B proteins.

A solubilized B70 protein or fragment of this invention can be used as an immunogen for the production of antisera or antibodies specific for the antigen or fragments thereof. The purified proteins can be used to screen monoclonal antibodies (mAbs) that bind B70, e.g., mAbs prepared by immunization with various forms of impure preparations containing the protein. In particular, the term "antibodies" also encompasses antigen-binding fragments of natural antibodies. The purified B70 proteins can also be used as a reagent to detect antibodies, e.g., endogenous antibodies, generated in response to the presence

of elevated levels of the marker or cell fragments containing the marker, both of which may be diagnostic of an abnormal or specific physiological or disease condition. Additionally, B70 fragments may also serve as immunogens to produce antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies raised against purified amino acid sequences encoded by nucleotide sequences described in SEQ ID NO: 1, or fragments of proteins containing them. In particular, this invention contemplates antibodies having binding affinity to or being raised against specific fragments which are predicted to lie outside of a lipid bilayer, preferably on the extracellular side.

The present invention contemplates the isolation of additional closely related species variants. Southern and Northern blot analysis should establish that similar genetic entities exist in other mammals. It is likely that the B70 proteins are widespread in various orders, e.g., rodents, lagomorphs, carnivores, artiodactyla, perissodactyla, and primates.

The invention also provides means to isolate a group of related proteins displaying both distinctness and similarities in structure, expression, and function. Elucidation of many of the physiological effects of the proteins will be greatly accelerated by the isolation and characterization of distinct molecular variants of B70. In particular, the present invention provides useful probes for identifying additional homologous genetic entities in different species.

The isolated genes will allow transformation of cells lacking expression of a corresponding B70 protein, e.g., either species types or cells which lack corresponding markers and exhibit negative background activity. Expression of transformed genes will allow isolation of antigenically pure cell lines, with defined or single species variants. This approach will allow for more sensitive detection and discrimination of the physiological effects of B70-interacting proteins. Subcellular fragments, e.g., cytoplasts or membrane fragments, can be isolated and used.

Dissection of the critical structural elements which effect the various biological functions provided by the B70 markers is possible using standard techniques of modern molecular biology, particularly in comparing members of the related class. See, e.g., the homolog-scanning mutagenesis technique described in Cunningham et al., (1989) Science 243:1339-1336; and approaches used in O'Dowd et al., (1988) J. Biol. Chem. 263:15985-15992; and Lechleiter et al., (1990) EMBO J. 9:4381-4390.

In particular, CD28/CTLA-4 binding segments of the type B antigens can be substituted between protein variants to determine what structural features are

important or critical in both type A partner binding affinity and specificity, as well as signal transduction. An array of different variants will be used to screen for those exhibiting combined properties of interaction with different species variants, e.g., with low or high species specificity.

Intracellular functions would probably involve segments of the type A binding partner which are normally accessible to the cytosol. Comparison of the signal transduction mediated by B7/BB1 binding with that mediated by B70 binding will be useful.

Expression and control of B70 protein exhibit cell specific differences. The controlling elements associated with the ligands exhibit differential developmental, tissue specific, or other expression patterns. Upstream or downstream genetic regions, e.g., control elements, are also made available.

Structural studies of B70 will lead to design of new related molecules, particularly functional analogs distinct from B7/BB1 exhibiting agonist or antagonist properties. This approach can be combined with previously described screening methods to isolate versions exhibiting desired spectra of activities.

Expression in different cell types will often result in glycosylation differences in a particular B70 antigen. Various protein variants may exhibit distinct functions based upon structural differences other than amino acid sequence. Differential modifications may be responsible for differential function, and elucidation of the effects is now made possible.

Thus, the present invention provides important reagents useful to study the relationship of B70 to the interaction between CTLA-4/CD28 and B7/BB1. Although the foregoing description has focused primarily upon human B70 protein, those of skill in the art will immediately recognize that the invention encompasses other variants, e.g., rat and other mammalian species or allelic variants, as well as variants thereof.

Antibodies

Antibodies can be raised to the various substantially purified B70 proteins, including species or allelic variants, and fragments thereof, both in their naturally occurring forms and in their recombinant forms. Additionally, antibodies can be raised to B70 proteins in either their active forms or in their inactive forms. Anti-idiotypic antibodies are also contemplated.

Antibodies, including binding fragments and single chain forms, against predetermined fragments of the B70 antigens can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins.

Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to B70 antigen, or screened for agonistic or antagonistic activity, e.g., neutralization of activity mediated through the CTLA-4/CD28 marker. These monoclonal antibodies will usually bind with a K_D of at most about 1 mM but more usually with stronger binding, e.g., with a K_D of at most about 300 µM, typically at most about 100 µM, more typically at most about 30 µM, preferably at most about 10 µM, and more preferably at most about 3 µM or better.

The antibodies, including antigen-binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to B70 and neutralize or block binding to type A binding partners, e.g., to inhibit the ability of B70 to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to toxins or radionuclides so that, when the antibody binds to B70, a cell expressing it (e.g., on its surface) is killed. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker, and may effect drug targeting.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they can be screened for ability to bind to the B70 antigens without inhibiting binding to type A binding partners, e.g., CTLA-4/CD28. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying B70 protein or its binding partners, e.g., CTLA-4/CD28.

B70 fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. A B70 protein or its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc.: see Microbiology, Hoeber Medical Division, Harper and Row, 1969; Landsteiner (1962) Specificity of Serological Reactions, Dover Publications, New York, and Williams et al., (1967) Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, New York, for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

It is sometimes desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice or other rodents, primates or other humans, etc. Descriptions of techniques for preparing such monoclonal antibodies may be

found in, e.g., Stites et al., (eds.) (1987) Basic and Clinical Immunology (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York; and particularly Kohler and Milstein (1975) in Nature 256:495-497, which discusses one method of generating monoclonal antibodies. Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma' that is capable of reproducing in vitro. The population of hybridomas is then screened to isolate individual clones, each of which secretes a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors: see Huse et al., (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda", Science 246:1275-1281; and Ward et al., (1989) Nature 341:544-546. The polypeptides and antibodies of the present invention may be used with or without modification, for example as chimeric or humanized antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced: see Cabilly, U.S. Patent No. 4,816,567; and Queen et al., (1989) Proc. Nat'l Acad. Sci. USA 86:10029-10033.

The antibodies of this invention can also be used for affinity chromatography in isolating the protein. A cell lysate can be passed through a column containing the antibodies linked to a solid support (e.g., particles) such as agarose, SEPHADEX (a beaded gel prepared by cross-linking dextran with epichlorhydrin under alkaline conditions), or the like. The column is then

washed and eluted with increasing concentrations of a mild denaturant, whereby the purified B70 protein will be released.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against a B70 protein will also be useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the respective antigens.

Making B70 Protein: Mimetics

DNA which encodes B70 protein or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or screening genomic libraries prepared from a wide variety of cell lines or tissue samples.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length B70 or fragments which can in turn be used e.g. to generate polyclonal or monoclonal antibodies; for binding studies; for construction and expression of modified molecules; and for structure/function studies. Each antigen or its fragments can be expressed in host cells that are transformed or transfected with appropriate expression vectors and can then be isolated from the cells or culture medium. These molecules can be produced free of protein and cellular contaminants that would normally accompany them in nature, and can then be purified free of protein or cellular contaminants derived from the recombinant host. In the pure state they are particularly useful in pharmaceutical compositions when combined with a pharmaceutically acceptable carrier and/or diluent. The antigen, or portions thereof, may be expressed as fusions with other proteins.

Expression vectors are typically self-replicating DNA or RNA constructs containing the desired antigen gene or its fragments, usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host. The specific type of control elements necessary to effect expression will depend upon the eventual host cell used. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that encodes a suitable

ribosome binding site, and sequences that terminate transcription and translation. Expression vectors also usually contain an origin of replication that allows the vector to replicate independently of the host cell.

Vectors of this invention contain DNA which encodes a B70 protein, or a fragment thereof, and will typically encode a biologically active polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such expression vectors which are capable of expressing eukaryotic cDNA coding for a B70 antigen in a prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the antigen is inserted into the vector such that growth of the host containing the vector expresses the cDNA in question. Usually, expression vectors are designed for stable replication in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not always necessary to require that an expression vector replicate in a host cell: e.g., it is possible to effect transient expression of the antigen or its fragments in various hosts using vectors that do not contain a replication origin that is recognized by the host cell. It is also possible to use vectors that cause integration of a B70 gene or its fragments into the host DNA by recombination, or to integrate a promoter which controls expression of an endogenous gene.

Vectors, as used herein, comprise plasmids, viruses, bacteriophages, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein: see, e.g., Pouwels et al., (1985 and Supplements) Cloning Vectors: A Laboratory Manual, Elsevier, NY., and Rodriguez et al., (1988) (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston, MA.

Transformed cells include cells, preferably mammalian, that have been transformed or transfected with vectors containing the B70 gene, which can be constructed using recombinant DNA techniques. For purposes of cloning, amplifying, and manipulating DNA encoding the protein, transformed host cells do not need to express the protein. However, they usually express the protein or its fragments, and this invention further contemplates culturing transformed cells in a nutrient medium, thus permitting the protein to accumulate in the culture. The protein can be recovered, either from the culture or from the culture medium.

For purposes of this invention, DNA sequences are operably linked when they function together. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, "operably linked" means "contiguous and in reading frame", however, certain genetic elements such as repressor genes are not contiguously linked but their encoded proteins still bind to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include both gram-negative and gram-positive organisms, e.g., *E. coli* and *B. subtilis*. Lower eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species of the genus *Dictyostelium*. Host cell lines of higher eukaryotes include established tissue culture cell lines from animal cells, which can be of non-mammalian origin, e.g., insect cells, and birds, or of mammalian origin, e.g., human, other primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, other and its vectors will be used generically to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express the B70 proteins or its fragments include, but are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540): see Brosius et al., (1988) "Expression Vectors Employing Lambda-, trp-, lac-, and Ipp-derived Promoters", in Rodriguez and Denhardt (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston, Chapter 10, pp. 205-236.

Lower eukaryotes, e.g., yeasts and *Dictyostelium*, may be transformed with vectors encoding the B70 protein sequence. For purposes of this invention, the most common lower eukaryotic host is baker's yeast, *Saccharomyces cerevisiae*. It will be used to represent lower eukaryotes generically, although a number of other strains and species are also available. Yeast vectors typically consist of a replication origin (except when the vector is of the integrating type), a selection gene, a promoter, DNA encoding the desired protein or its fragments, and sequences for termination of translation, polyadenylation, and termination of transcription. Suitable promoters for expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other

glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase-2 promoter or metallothionine promoter. Suitable vectors include derivatives of the following types: self-replicating low-copy-number (such as the YRp-series), self-replicating high-copy-number (such as the YEp-series), integrating types (such as the YIp-series), and mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are the preferred host cells for expression of the functionally active B70 antigen. In principle, many higher eukaryotic tissue culture cell lines are workable, e.g., insect baculovirus expression systems, whether from an invertebrate or vertebrate source. However, mammalian cells are preferred. Transformation or transfection and propagation of such cells has become a routine procedure. Examples of useful cell lines include HeLa cells, Chinese hamster ovary cell lines, baby rat kidney cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a site for initiation of translation, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a site for termination of transcription. These vectors also usually contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Representative examples of suitable expression vectors include pCDNA1; pCD [see Okayama et al., (1985) Mol. Cell Biol. 5:1136-1142]; pMC1neo Poly-A [see Thomas et al., (1987) Cell 51:503-512]; and a baculovirus vector such as pAC 373 or pAC 610.

It will often be desired to express a B70 protein or polypeptide in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, a B70 gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes. Using this approach, certain mammalian glycosylation patterns will be achievable or approximated in prokaryote or other cells.

The B70 protein, or a fragment thereof, may be engineered to be linked through phosphatidyl inositol to a cell membrane, but can be removed from membranes by treatment with a phosphatidyl inositol cleaving enzyme, e.g., phosphatidyl inositol phospholipase-C. This releases the antigen in a biologically active form, and allows purification by standard procedures of

protein chemistry: see, e.g., Low (1989) Biochim. Biophys. Acta 988:427-454; Tse et al., (1985) Science 230:1003-1008; and Brunner et al., (1991) J. Cell Biol. 114:1275-1283.

Now that the B70 protein has been characterized, fragments or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky (1984) The Practice of Peptide Synthesis, Springer-Verlag, New York; and Bodanszky (1984) The Principles of Peptide Synthesis, Springer-Verlag, New York. For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, using a 4-nitrophenyl ester, N-hydroxy-succinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

The B70 protein, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide fragments to the terminal amino acid. Amino groups that are not being used in the coupling reaction are typically protected to prevent coupling at an incorrect location.

If a solid phase synthesis is adopted, the C-terminal amino acid is bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as it has a binding capability to a reactive carboxyl group. Examples of such insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, tert-alkyloxycarbonyl-hydrazidated resins, and the like.

An amino acid protected at the amino group is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step-by-step. After the complete sequence has been synthesized, the peptide is split off from the insoluble carrier. This solid-phase approach is generally described by Merrifield et al., (1963) in J. Am. Chem. Soc. 85:2149-2156.

The prepared B70 antigen and fragments thereof can be isolated and purified from the reaction mixture by means of peptide separation, for example, by extraction, precipitation, electrophoresis and various forms of chromato-

graphy, and the like. The B70 antigens of this invention can be obtained in varying degrees of purity depending upon their intended use. Purification can be accomplished by use of the protein purification techniques disclosed herein or by the use of the antibodies herein described in immunoabsorbant affinity chromatography. This immunoabsorbant affinity chromatography is carried out by first linking the antibodies to a solid support and then contacting the linked antibodies with solubilized lysates of appropriate source cells, lysates of other cells expressing the protein, or lysates or supernatants of cells producing the B70 protein as a result of DNA techniques (see below).

Uses

The present invention provides reagents which will find use in diagnostic applications as described elsewhere herein, e.g., in the general description for developmental abnormalities, or below in the description of kits for diagnosis.

This invention also provides reagents with significant therapeutic value either in vitro or in vivo. The B70 antigen (naturally occurring or recombinant), fragments thereof and antibodies thereto, along with compounds identified as having binding affinity to B70 protein, e.g., type A binding partners/antigens, should be useful in the treatment of conditions associated with abnormal physiology or development, including abnormal proliferation, e.g., cancerous conditions, or degenerative conditions, particularly those involving the immune system. In particular, agonists of B70 will substantially augment T cell responses. This may be useful, e.g., in vaccine reactions, in tumor suppression or rejection, or in ameliorating immunodeficient conditions. In other situations, an antagonist may block an inappropriate immune response, e.g., in autoimmunity, hypersensitivity, or tissue transplantation, where an immune response may need to be down-regulated. Such effects may find use in either an in vitro context, or an in vivo context.

In particular, CD28 interacts with B7 to costimulate T cell activation initiated through the CD3/TcR complex: see Linsley et al., (1993) Ann. Rev. Immunol. 11:191-212. Interaction between B7 and CD28 also is involved in cell-mediated immune responses, e.g., allogeneic responses, B-cell-stimulated T-cell responses, and Ig production stimulated by T cells: see also Paul (ed.) (1993) Fundamental Immunology (3rd ed.) Raven Press, New York, NY. Partner binding to CD28 activates (1) expression of cytokines, e.g., IL-2, IFN-γ, TNF-α, lymphotoxin, GM-CSF, and IL-3; and (2) differentiation to cytotoxic cells. The enhanced cytokine expression induced by stimulation via CD28 appears to

result from prolonged message lifetime leading to higher expression, and causes the T helper subset activities. The activation of cytotoxicity results in potent tumor cell killing. Since B70 appears to act through the same structural CD28/CTLA-4 antigen marker as B7/BB1, agonists and antagonists of B70 should exhibit similar biological properties to corresponding molecules which act on the B7 antigen.

Thus, B70 homologues should be useful, alone or in combination with B7/BB1 homologues, to modulate mitogenic activity, to regulate lymphokine secretion, and to modulate cell-mediated cytotoxicity. Medical conditions where such intervention would be indicated are described, e.g., in Berkow (ed.) (1987) The Merck Manual Merck & Co., Rahway, N.J. These effects may require costimulatory signals via the TcR or CD3 markers.

Recombinant B70 or antibodies against B70 can be purified and then administered to appropriate in vitro cultures, or to a patient. These reagents can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, along with physiologically innocuous stabilizers and excipients. Combinations of B70 and B7/BB1 agonists or antagonists may be particularly useful. These combinations can be sterile-filtered and placed into dosage forms, e.g., by lyophilization in dosage vials, or be stored in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof, including forms which are do not bind to complement.

Drug screening using CTLA-4/CD28 or fragments thereof can be performed to identify compounds having binding affinity to B70 protein, including isolation of naturally associated components. Subsequent biological assays can then be utilized to determine if the compound has intrinsic stimulating activity and is therefore a blocker or antagonist in that it blocks or neutralizes the activity of B70. Likewise, a compound other than B7/BB1 but having intrinsic stimulating activity can activate the type A binding partner/antigen, e.g., CTLA-4/CD28, and is thus an agonist in that it simulates or enhances the activity of B70 antigen. This invention further contemplates the therapeutic use of neutralizing antibodies to B70 as antagonists. This approach should be particularly useful with other B70 protein variants.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used in vitro may provide useful guidance in the amounts useful for

in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman et al., (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn.; Avis et al. (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications, Dekker, NY; Lieberman et al., (eds.) (1990) Pharmaceutical Dosage Forms: Tablets, Dekker, NY; and Lieberman et al., (eds.) (1990) Pharmaceutical Dosage Forms, Dekker, NY. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey. Dosage ranges would ordinarily be expected to be in amounts lower than about 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations or a slow release apparatus will often be utilized for continuous administration: see, e.g., Langer (1990) Science 249:1527-1533.

B70 protein, fragments thereof, and antibodies to it or to its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, may be conjugated to carrier proteins such as ovalbumin or serum albumin prior to their administration. The compounds may be administered as active ingredients in any conventional formulation, e.g., in a dosage unit. Whereas it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation. Formulations typically comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Combinations with B7/BB1 or with reagents thereto, e.g., agonists or antagonists, are contemplated. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy: see, e.g., Gilman et al., (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon

Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn. The therapeutic compositions of this invention may be combined with or used in association with other biologically active agents.

Both the naturally occurring and the recombinant form of the B70 proteins of this invention are particularly useful in kits and assay methods which are capable of screening compounds for binding activity to the proteins. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period, particularly diversity combinatorial libraries. See, e.g., Fodor et al., (1991) Science 251:767-773, which describes means for testing of binding affinity by a plurality of defined polymers synthesized on a solid substrate. The development of suitable assays can be greatly facilitated by the availability of large amounts of purified, soluble B70 antigen as provided by this invention.

For example, antagonists can normally be found once the antigen has been structurally defined. Testing of potential antigen analogs is now possible upon the development of highly automated assay methods. In particular, new agonists and antagonists will be discovered by using screening techniques described herein. Of particular importance are compounds found to have a combined binding affinity for multiple B70 binding partners, e.g., compounds which can serve as antagonists for variants of the B70 protein.

This invention is particularly useful for screening compounds by using recombinant protein in any of a variety of drug screening techniques. The advantages of using a recombinant protein in screening for specific binding proteins include: (a) improved renewable source of B70 from a specific source; (b) potentially greater number of proteins per cell giving better signal-to-noise ratio in assays; and (c) species variant specificity (theoretically giving greater biological and disease specificity).

One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant DNA molecules expressing type A antigens/binding partners. Cells may be isolated which express B70 in isolation from any others. Such cells, either in viable or fixed form, can be used for standard binding-partner assays. See also Parce et al., (1989) Science 246:243-247; and Owicki et al., (1990) Proc. Nat'l Acad. Sci. USA 87:4007-4011, which describe sensitive methods to detect cellular responses. Competitive assays are particularly useful, where the cells (source of B70 protein) are contacted and incubated with a labeled CTLA-4/CD28 or antibody having known binding affinity to B70, such as ^{125}I-antibody, and a test sample

whose binding affinity to the binding composition is being measured. The bound and free labeled binding partners for B70 are then separated to assess the degree of B70 binding. The amount of test compound bound is inversely proportional to the amount of labeled CTLA-4/CD28 binding to the known source. Any one of numerous techniques can be used to separate bound from free antigen to assess the degree of binding. This separation step could typically involve a procedure such as adhesion to filters followed by washing, adhesion to plastic followed by washing, or centrifugation of the cell membranes. Viable cells could also be used to screen for the effects of drugs on cellular functions mediated by interaction of B70 with CTLA-4/CD28, which may involve, e.g., second messenger levels, i.e., Ca^{++}; cell proliferation; inositol phosphate pool changes; and others. Some detection methods allow for elimination of a separation step, e.g., a proximity-sensitive detection system. Calcium-sensitive dyes will be useful for detecting Ca^{++} levels, with a fluorimeter or a fluorescence cell sorting apparatus.

Another method utilizes membranes from transformed eukaryotic or prokaryotic host cells as the source of the B70 antigen. These cells are stably transformed with DNA vectors directing the expression of a B70 antigen in e.g. a membrane-bound form. Essentially, the membranes would be prepared from the cells and used in a type A/type B binding assay such as the competitive assay set forth above.

Still another approach is to use solubilized, unpurified or solubilized, purified B70 protein from transformed eukaryotic or prokaryotic host cells. This allows for a "molecular" binding assay with the advantages of increased specificity, the ability to automate, and high drug test throughput.

Another technique for drug screening involves an approach which provides high throughput screening for new compounds having suitable binding affinity, e.g., to a CTLA-4/CD28 antigen, and is described in detail in International Patent Application no. WO 84/03564 (Commonwealth Serum Labs.), published on September 13 1984. First, large numbers of different small peptide test compounds are synthesized on a solid substrate, e.g., plastic pins or some other appropriate surface; see Fodor et al. (1991). Then all the pins are reacted with solubilized type A antigen, unpurified or purified, and washed. The next step involves detecting bound binding partner for the type A binding partner. New functional equivalents for B7/BB1 or B70 should be detected.

Rational drug design may also be based upon structural studies of the molecular shapes of the B70 antigen and other effectors or analogs. Effectors may be other proteins which mediate other functions in response to partner

binding, or other proteins which normally interact with a type A binding partner. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., X-ray crystallography or two-dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York.

Purified B70 or CTLA-4/CD28 antigen can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to these antigens can be used as capture antibodies to immobilize the respective antigen on the solid phase.

Kits

This invention also contemplates use of B70 antigens, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of B70 or a binding partner for B70. Typically the kit will have a compartment containing either a defined B70 protein peptide or gene segment or a reagent which recognizes one or the other, e.g., type A binding partners or antibodies.

A kit for determining the binding affinity of a test compound to a B70 protein will typically comprise a test compound; a labeled compound, e.g., a type A binding partner such as CTLA-4/CD28 or an antibody having known binding affinity for B70; a source of B70 antigen (naturally occurring or recombinant); and a means for separating bound from free labeled compound, such as a solid phase for immobilizing the antigen. Once compounds are screened, those having suitable binding affinity to the antigen can be evaluated in suitable biological assays as are well known in the art, to determine whether they act as agonists or antagonists of B70 functions. The availability of recombinant B70 polypeptides also provides well definable standards for calibrating such assays.

A preferred kit for determining the concentration of, e.g., a B70 antigen in a sample would typically comprise a labeled compound, e.g., type A binding partner or antibody, having known binding affinity for the B70 antigen, a source of antigen (naturally occurring or recombinant), and a means for separating bound from free labeled compound, for example, a solid phase for immobilizing the B70 antigen. Compartments containing reagents, and instructions, will normally be provided.

Antibodies, including antigen-binding fragments, specific for the B70 antigen, or type A binding partners, are useful in diagnostic applications to detect the presence of elevated levels of B70 antigen and/or its fragments. Such diagnostic assays can employ lysates, live cells, fixed cells, immunofluorescence, cell cultures, or body fluids, and further can detect antigens related to the antigen in serum, or the like. Diagnostic assays may be homogeneous (without a separation step between free reagent and type A/type B binding partner complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay, enzyme-multiplied immunoassay technique, substrate-labeled fluorescent immunoassay, and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to a B70 antigen or to a particular fragment thereof. Similar assays have also been extensively discussed in the literature: see, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH; Van Vunakis et al., (1990) Meth. Enzymol. 70:1-525; and Coligan et al., (eds.) (1993) Current Protocol in Immunology, Greene/Wiley, NY.

Anti-idiotypic antibodies may have similar use to diagnose presence of antibodies against a B70 antigen, since they may be diagnostic of various abnormal states. For example, overproduction of B70 antigen may result in various immunological reactions which may be diagnostic of abnormal physiological states, particularly in proliferative cell conditions such as cancer or abnormal differentiation.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. Depending upon the nature of the assay for use with a kit of the present invention, its protocol and its label, labeled or unlabeled antibody, labeled or unlabeled binding partner, or labeled B70 antigen, is provided – usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent. Desirably, the reagents are provided as a dry lyophilized powder, which may be reconstituted in an aqueous medium providing appropriate concentrations of reagents for performing the assay.

Any of the aforementioned constituents of the drug screening and the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labeling may be achieved by covalently or non-covalently attaching a moiety which directly or indirectly provides a detectable

signal. In any of these assays, the test compound, antigen, or antibodies thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labeling include biotinylation of one constituent, followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating the bound from the free marker/antigen, or alternatively the bound from the free test compound. The B70 antigen can be immobilized on various matrixes followed by washing. Suitable matrixes include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the B70 antigen to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin. The last step in this approach involves the precipitation of type A/type B binding complex by any of several methods, e.g., using an organic solvent such as polyethylene glycol or a salt such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein-antibody magnetizable particle method described in Rattle et al., (1984) Clin. Chem. 30:1457-1461, and the double-antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678.

The methods for linking proteins or their fragments to the various labels have been extensively reported in the literature and do not require detailed discussion here. For example, an activated carboxyl group, generated e.g. by means of a carbodiimide, or active esters, can be used to form peptide bonds; the reaction of a mercapto group with an activated halocompound such as chloroacetyl compound can be used to yield thioethers; or an activated olefin such as maleimide can be used for linkage. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences which encode a B70 antigen or hybridize to such genes, including antisense sequences. These sequences can be used as probes for detecting levels of message in samples from patients suspected of having an abnormal condition, e.g., cancer or developmental problem, characterized by abnormal antigen, either qualitatively or quantitatively. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide

probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that, upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid-released translation, and hybrid-arrested translation. This also includes amplification techniques such as PCR.

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers: see, e.g., Viallet et al., (1989) Progress in Growth Factor Res. 1:89-97.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to specific embodiments.

EXAMPLES

General Methods

Some of the standard methods are described or referenced, e.g., in Maniatis et al., (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols. 1-3, CSH Press, NY; Ausubel et al., Biology, Greene Publishing Associates, Brooklyn, NY; or Ausubel et al., (1987 and Supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York; Innis et al., (eds.) (1990) PCR Protocols: A Guide to Methods and Applications Academic Press, NY. Methods for protein purification

include such methods as precipitation with ammonium sulfate, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel et al., (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in Methods in Enzymology, vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia (Piscataway, N.J.) or Bio-Rad (Richmond, CA). Combination with recombinant techniques allows fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence: see, e.g., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, NY.; and Crowe et al., (1992) QIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, CA.

FACS analyses are described in Melamed et al., (1990) Flow Cytometry and Sorting Wiley-Liss, Inc., New York, NY; Shapiro (1988) Practical Flow Cytometry Liss, New York, NY; and Robinson et al., (1993) Handbook of Flow Cytometry Methods Wiley-Liss, New York, NY.

Expression Cloning and Sequencing of the B70 Antigen

A cDNA encoding a B70 antigen was cloned by expression cloning using mAb IT2.236 to select COS7 cells transfected with a cDNA library cloned into the *Bst*XI-*Not*I sites of the pJFE14 vector: see Aruffo and Seed (1987) Proc. Nat'l Acad. Sci. USA 84:8573-8577. The library was made from RNA isolated from a human EBV-transformed B-lymphoblastoid cell line JY (cell line generously provided by Dr. H. Spits, Netherlands Cancer Institute; Amsterdam).

Preparation of neutralizing mAb

IT2 monoclonal antibodies, IT2.a, IgG1; and IT2.b, IgG2b, were generated by fusing myeloma cells with splenocytes from BALB/c mice immunized with JY B lymphoblastoid cells (which are readily available). A 4-hour ^{51}Cr-radioisotope release assay was used to screen hybridoma supernatants for the ability to block the killing of JY cells by YT2C2 in the presence of saturating concentrations of anti-B7 mAb L307: see Azuma et al., (1992) J. Immunol. 149:1115-1123. YT2C2 expresses CD28 but not CTLA-4, indicating that the antigen recognized by IT2 monoclonal antibodies to B70 functionally binds to CD28 that is expressed on JY cells.

Anti-B7 mAb (L307, murine IgG1) and other anti-human leukocyte antigen mAbs were obtained from Becton-Dickinson. A mouse CTLA-4 human IgG fusion protein was obtained from Dr. Peter Lane (Basel Institute). FITC-conjugated goat anti-human IgG (mouse absorbed) was purchased from CalTag (Burlingame, CA). PBMC were isolated from healthy human volunteers by using Ficoll-Hypaque density gradients. Freshly isolated PBMC were cultured in medium containing 100 U/ml rIL-2 for 10 days in tissue-culture flasks coated with purified anti-CD3 mAb. PBL were stained with biotin-conjugated IT2 or biotin-conjugated anti-B7 (L307) together with FITC anti-CD14 (Leu M3), FITC anti-CD19 (Leu 12), or FITC anti-CD3 (Leu 4) followed by PE-streptavidin. Immunofluorescence and flow cytometry were performed using a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA) as described in Lanier and Recktenwald (1991) Methods: A Companion to Methods in Enzymology 2:192-199. For two-color immunofluorescence, an electronic gate was set on FITC-stained CD3-, CD14-, or CD19-positive cells to identify T cells, monocytes, or B cells, respectively, and PE labeled anti-B7 and anti-B70 fluorescence were evaluated. For CTLA-4-Ig binding experiments, JY or B7+ P815 were stained with saturating amounts of irrelevant isotype-matched control mAb, L307 (anti-B7, IgG1), IT2 (IgG1), or a mixture of IT2 and L307 for 20 min before the addition of CTLA-4-Ig. Cells were washed and stained with FITC conjugated goat anti-human IgG (absorbed to remove reactivity with murine mAb).

Distribution of B70 antigen

Flow cytometric analysis revealed that B70 was highly expressed on several types of human B lymphoma cell lines, e.g., JY, WA, NC37, RPMI-8866, Daudi, and Raji, whereas resting peripheral blood B cells and pre-B leukemia cell lines, e.g., NALM6, SMS-SB, 697, expressed only low levels of B7 or B70. B70, like B7, is induced after lymphocyte activation. Peripheral blood B cells stimulated via CD40 cross-linking express significantly higher levels of B7 or B70. LPS- or PWM-activated B cells and freshly isolated tonsilar B cells ressected with chronic tonsillitis express significant levels of B70, as well as B7.

Whereas peripheral blood T cells were negative, both B7 and B70 were induced after 10-day stimulation with immobilized anti-CD3 mAb and rIL-2. $CD4^+$ T cell clones, $CD8^+$ T cell clones, and NK clones expressed B70 as well as B7, whereas T cell leukemia cells lines, e.g., Jurkat, Molt 4, PEER, CEM, HUT-78, did not express significant amounts of these antigens.

Although B7 and B70 are apparently co-expressed by normal activated T and B lymphocytes, their expression differs in myeloid cells. Freshly isolated peripheral blood monocytes constitutively express significant levels of B70, with only much lower amounts of B7. After incubation for 24 hours with γ-interferon, B70 on monocytes was extremely enhanced. Peripheral blood dendritic cells expressed B7 and did express ten-fold higher amounts of B70 than B7. In tissue sections of normal skin, epidermal Langerhans' cells were strongly reactive with anti-B70 and anti-B7 monoclonal antibodies.

Function of B70 antigen

To examine the function of B70, freshly isolated nonadherent PBL were co-cultured with irradiated allogeneic PBMC in a 7-day Mixed Lymphocyte Reaction (MLR). In essence, IT2 monoclonal antibodies completely inhibited the proliferative response, comparable to inhibition with anti-CD28 monoclonal antibody. By contrast, anti-B7 monoclonal antibody L307, alone, only partially inhibited alloantigen-induced MLR. Relative affinity of the anti-B7 versus anti-B70 monoclonal antibody is unlikely to explain the inability of anti-B7 to inhibit primary allogeneic MLR using irradiated PBMC as stimulators since it has previously been shown that L307 efficiently blocks MLR when human B7 transfectants are used as stimulators. Therefore, B70 present on monocytes may initially interact with CD28 on resting T cells to initiate the allogeneic MLR. Cytokine production, e.g., γ-interferon, during the response may up-regulate B7, and that, in turn, may amplify the response.

Immunoprecipitation of B70

Viable JY cells were surface-biotinylated, lysed, and immunoprecipitated using control Ig, IT2 mAb, and anti-B7 mAb (L307). Samples were resuspended in sample buffer with or without 2-mercaptoethanol and analyzed by SDS-PAGE on 10% acrylamide gels. Viable NK cell clones were surface-labeled with ^{125}I, and lysed in 1% NP-40 TRIS-buffered saline, and antigens were immunoprecipitated using Pansorbin (Calbiochem, San Diego, CA) coated with rabbit anti-mouse Ig and IT2 or anti-B7 (L307), as described by Lanier et al., (1988) J. Immunol 141:3478-3482.

A single glycoprotein of MW of approximately 70 kD was immunoprecipitated from biotin or ^{125}I-surface-labeled JY cells. Consistent with prior reports, anti-B7 monoclonal antibody immunoprecipitated a lower MW

protein of 55-60 kD. Sequential immunoprecipitation indicated that B7 and B70 were independent molecules. Preclearing the lysates with anti-B7 monoclonal antibody completely depleted all B7 glycoprotein, but failed to remove any B70. CTLA-4 Ig immunoprecipitated two distinct molecules of approximately 60 kD (assumed to be B7) and 70 kD (assumed to be B70).

To exclude differential glycosylation, affinity purified B7 and B70 glycoproteins were treated with 15 U/ml recombinant N-glycanase F and analyzed by SDS-PAGE. An approximately 40 kD polypeptide was revealed after deglycosylation of B70 compared to a 30 kD deglycosylated B7 polypeptide. These results suggest that the B70 polypeptide is larger than B7 and represents a unique CTLA-4 binding ligand.

Source of B70 Antigen

Multiple cell lines were screened for one expressing a B70 antigen. Expression may be based either upon protein expression, or, in certain contexts, upon mRNA expression. JY, an EBV-transformed B lymphoblastoid cell line, was suspected to express a novel binding partner for CD28/CTLA-4. Anti-B7 failed to completely block binding of CTLA-4-Ig to these cells. Moreover, the IT2.236 antibody (see Aruffo and Seed (1987) Proc. Nat'l Acad. Sci. USA 84:8573-8577) labeled this cell line. COS7 cells were also transfected with an expression vector LL278 containing B70 cDNA, and were subsequently stained with one of the IT2 monoclonal antibodies (X, IT2.236) to B70 (Figure 1A), with CTLA-Ig fusion (Y, Figure 1B), or with IT2.236 (Z, Figure 1C). In all these experiments, results with a control are indicated by Q. This experiment shows that the B70 transfection was efficient and that the anti-B70 monoclonal antibody IT2.236 was specific for B70 antigen.

The CTLA-Ig fusion protein was prepared by the method of Lane et al., Immunol. Vol. 80:1 (September 1993), pages 56-61.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

SEQUENCE LISTING

SEQ ID NO: 1 is B70 cDNA nucleic acid sequence.
SEQ ID NO: 2 is B70 predicted amino acid sequence.

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Schering Corporation
(B) STREET: 2000 Galloping Hill Road
(C) CITY: Kenilworth
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 07033
(G) TELEPHONE: 201-822-7000
(H) TELEFAX: 201-822-7039
(I) TELEX: 219165

(ii) TITLE OF INVENTION: Purified Mammalian CTLA-4 Binding Protein and Related Reagents

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Schering-Plough Corporation, M-3-W
(B) STREET: One Giralda Farms
(C) CITY: Madison
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07940-1000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh IIci
(C) OPERATING SYSTEM: System Software 7.1
(D) SOFTWARE: Microsoft Word 5.1a

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION DATA: PCT/US 94/
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/120,606
(B) FILING DATE: 13-SEP-1993

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/116,882
(B) FILING DATE: 03-SEP-1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Blasdale, John H. C.
(B) REGISTRATION NUMBER: 31,895
(C) REFERENCE/DOCKET NUMBER: DX0390K1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-822-7398
(B) TELEFAX: 201-822-7039

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1428 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 152..1123

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAAGAGGAGC CTTAGGAGGT ACGGGGAGCT CGCAAATACT CCTTTTGGTT TATTCTTACC      60

ACCTTGCTTC TGTGTTCCTT GGGAATGCTG CTGTGCTTAT GCATCTGGTC TCTTTTTGGA     120

GCTACAGTGG ACAGGCATTT GTGACAGCAC T ATG GGA CTG AGT AAC ATT CTC         172
                                  Met Gly Leu Ser Asn Ile Leu
                                    1               5

TTT GTG ATG GCC TTC CTG CTC TCT GGT GCT GCT CCT CTG AAG ATT CAA       220
Phe Val Met Ala Phe Leu Leu Ser Gly Ala Ala Pro Leu Lys Ile Gln
          10                  15                  20

GCT TAT TTC AAT GAG ACT GCA GAC CTG CCA TGC CAA TTT GCA AAC TCT       268
Ala Tyr Phe Asn Glu Thr Ala Asp Leu Pro Cys Gln Phe Ala Asn Ser
      25                  30                  35

CAA AAC CAA AGC CTG AGT GAG CTA GTA GTA TTT TGG CAG GAC CAG GAA       316
Gln Asn Gln Ser Leu Ser Glu Leu Val Val Phe Trp Gln Asp Gln Glu
  40                  45                  50                  55

AAC TTG GTT CTG AAT GAG GTA TAC TTA GGC AAA GAG AAA TTT GAC AGT       364
Asn Leu Val Leu Asn Glu Val Tyr Leu Gly Lys Glu Lys Phe Asp Ser
                  60                  65                  70

GTT CAT TCC AAG TAT ATG GGC CGC ACA AGT TTT GAT TCG GAC AGT TGG       412
Val His Ser Lys Tyr Met Gly Arg Thr Ser Phe Asp Ser Asp Ser Trp
              75                  80                  85

ACC CTG AGA CTT CAC AAT CTT CAG ATC AAG GAC AAG GGC TTG TAT CAA       460
Thr Leu Arg Leu His Asn Leu Gln Ile Lys Asp Lys Gly Leu Tyr Gln
          90                  95                  100
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TGT ATC ATC CAT CAC AAA AAG CCC ACA GGA ATG ATT CGC ATC CAC CAG      508
Cys Ile Ile His His Lys Lys Pro Thr Gly Met Ile Arg Ile His Gln
    105                 110                 115

ATG AAT TCT GAA CTG TCA GTG CTT GCT AAC TTC AGT CAA CCT GAA ATA      556
Met Asn Ser Glu Leu Ser Val Leu Ala Asn Phe Ser Gln Pro Glu Ile
120                 125                 130                 135

GTA CCA ATT TCT AAT ATA ACA GAA AAT GTG TAC ATA AAT TTG ACC TGC      604
Val Pro Ile Ser Asn Ile Thr Glu Asn Val Tyr Ile Asn Leu Thr Cys
                140                 145                 150

TCA TCT ATA CAC GGT TAC CCA GAA CCT AAG AAG ATG AGT GTT TTG CTA      652
Ser Ser Ile His Gly Tyr Pro Glu Pro Lys Lys Met Ser Val Leu Leu
            155                 160                 165

AGA ACC AAG AAT TCA ACT ATC GAG TAT GAT GGT ATT ATG CAG AAA TCT      700
Arg Thr Lys Asn Ser Thr Ile Glu Tyr Asp Gly Ile Met Gln Lys Ser
        170                 175                 180

CAA GAT AAT GTC ACA GAA CTG TAC GAC GTT TCC ATC AGC TTG TCT GTT      748
Gln Asp Asn Val Thr Glu Leu Tyr Asp Val Ser Ile Ser Leu Ser Val
    185                 190                 195

TCA TTC CCT GAT GTT ACG AGC AAT ATG ACC ATC TTC TGT ATT CTG GAA      796
Ser Phe Pro Asp Val Thr Ser Asn Met Thr Ile Phe Cys Ile Leu Glu
200                 205                 210                 215

ACT GAC AAG ACG CGG CTT TTA TCT TCA CCT TTC TCT ATA GAG CTT GAG      844
Thr Asp Lys Thr Arg Leu Leu Ser Ser Pro Phe Ser Ile Glu Leu Glu
                220                 225                 230

GAC CCT CAG CCT CCC CCA GAC CAC ATT CCT TGG ATT ACA GCT GTA CTT      892
Asp Pro Gln Pro Pro Pro Asp His Ile Pro Trp Ile Thr Ala Val Leu
            235                 240                 245

CCA ACA GTT ATT ATA TGT GTG ATG GTT TTC TGT CTA ATT CTA TGG AAA      940
Pro Thr Val Ile Ile Cys Val Met Val Phe Cys Leu Ile Leu Trp Lys
        250                 255                 260

TGG AAG AAG AAG AAG CGG CCT CGC AAC TCT TAT AAA TGT GGA ACC AAC      988
Trp Lys Lys Lys Lys Arg Pro Arg Asn Ser Tyr Lys Cys Gly Thr Asn
    265                 270                 275

ACA ATG GAG AGG GAA GAG AGT GAA CAG ACC AAG AAA AGA GAA AAA ATC     1036
Thr Met Glu Arg Glu Glu Ser Glu Gln Thr Lys Lys Arg Glu Lys Ile
280                 285                 290                 295

CAT ATA CCT GAA AGA TCT GAT GAA GCC CAG CGT GTT TTT AAA AGT TCG     1084
His Ile Pro Glu Arg Ser Asp Glu Ala Gln Arg Val Phe Lys Ser Ser
                300                 305                 310

AAG ACA TCT TCA TGC GAC AAA AGT GAT ACA TGT TTT TAATTAAAGA          1130
Lys Thr Ser Ser Cys Asp Lys Ser Asp Thr Cys Phe
            315                 320
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GTAAAGCCCA TACAAGTATT CATTTTTTCT ACCCTTTCCT TTGTAAGTTC CTGGGCAACC 1190

TTTTTGATTT CTTCCAGAAG GCAAAAAGAC ATTACCATGA GTAATAAGGG GGCTCCAGGA 1250

CTCCCTCTAA GTGGAATAGC CTCCCTGTAA CTCCAGCTCT GCTCCGTATG CCAAGAGGAG 1310

ACTTTAATTC TCTTACTGCT TCTTTTCACT TCAGAGCACA CTTATGGGCC AAGCCCAGCT 1370

TAATGGCTCA TGACCTGGAA ATAAAATTTA GGACCAATAA AAAAAAAAAA AAAAAAAA 1428

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 323 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Leu Ser Asn Ile Leu Phe Val Met Ala Phe Leu Leu Ser Gly
1 5 10 15

Ala Ala Pro Leu Lys Ile Gln Ala Tyr Phe Asn Glu Thr Ala Asp Leu
20 25 30

Pro Cys Gln Phe Ala Asn Ser Gln Asn Gln Ser Leu Ser Glu Leu Val
35 40 45

Val Phe Trp Gln Asp Gln Glu Asn Leu Val Leu Asn Glu Val Tyr Leu
50 55 60

Gly Lys Glu Lys Phe Asp Ser Val His Ser Lys Tyr Met Gly Arg Thr
65 70 75 80

Ser Phe Asp Ser Asp Ser Trp Thr Leu Arg Leu His Asn Leu Gln Ile
85 90 95

Lys Asp Lys Gly Leu Tyr Gln Cys Ile Ile His His Lys Lys Pro Thr
100 105 110

Gly Met Ile Arg Ile His Gln Met Asn Ser Glu Leu Ser Val Leu Ala
115 120 125

Asn Phe Ser Gln Pro Glu Ile Val Pro Ile Ser Asn Ile Thr Glu Asn
130 135 140

Val Tyr Ile Asn Leu Thr Cys Ser Ser Ile His Gly Tyr Pro Glu Pro
145 150 155 160

Lys Lys Met Ser Val Leu Leu Arg Thr Lys Asn Ser Thr Ile Glu Tyr
165 170 175

Asp Gly Ile Met Gln Lys Ser Gln Asp Asn Val Thr Glu Leu Tyr Asp
180 185 190

Val Ser Ile Ser Leu Ser Val Ser Phe Pro Asp Val Thr Ser Asn Met
195 200 205

Thr Ile Phe Cys Ile Leu Glu Thr Asp Lys Thr Arg Leu Leu Ser Ser
210 215 220

Pro Phe Ser Ile Glu Leu Glu Asp Pro Gln Pro Pro Pro Asp His Ile
225 230 235 240

Pro Trp Ile Thr Ala Val Leu Pro Thr Val Ile Ile Cys Val Met Val
245 250 255

Phe Cys Leu Ile Leu Trp Lys Trp Lys Lys Lys Lys Arg Pro Arg Asn
260 265 270

Ser Tyr Lys Cys Gly Thr Asn Thr Met Glu Arg Glu Glu Ser Glu Gln
275 280 285

Thr Lys Lys Arg Glu Lys Ile His Ile Pro Glu Arg Ser Asp Glu Ala
290 295 300

Gln Arg Val Phe Lys Ser Ser Lys Thr Ser Ser Cys Asp Lys Ser Asp
305 310 315 320

Thr Cys Phe

CLAIMS:

1. A protein comprising a B70 antigen sequence or a peptide of said B70 antigen.

2. A fusion protein comprising a B70 antigen sequence.

3. A protein or peptide as claimed in Claim 1, selected from the group consisting of:

a) a peptide from a warm-blooded animal selected from the group of birds and mammals;
b) a peptide comprising at least one polypeptide segment of SEQ ID NO: 2;
c) a peptide which exhibits a post-translational modification pattern distinct from natural B70 antigen; and
d) a peptide which binds to CTLA-4 or CD28.

4. A protein or peptide as claimed in Claim 3, wherein said warm-blooded animal is a rodent or primate.

5. A substantially pure B70 antigen or peptide thereof as claimed in Claim 1.

6. A nucleic acid exhibiting homology to a sequence encoding the B70 antigen sequence or a peptide of said B70 antigen as claimed in Claim 1.

7. A nucleic acid as claimed in Claim 6 encoding a fusion protein comprising the B70 antigen sequence.

8. A nucleic acid as claimed in Claim 6 encoding a protein or peptide selected from the group consisting of:

a) a peptide from a warm-blooded animal selected from the group of birds and mammals;
b) a peptide comprising at least one polypeptide segment of SEQ ID NO: 2;
c) a peptide which exhibits a post-translational modification pattern distinct from natural B70 antigen; and
d) a peptide which binds to CTLA-4 or CD28.

9. A nucleic acid as claimed in Claim 8, encoding a protein or peptide from a warm-blooded animal that is a rodent or primate.

10. A nucleic acid as claimed in Claim 6 encoding a B70 protein.

11. A nucleic acid as claimed in Claim 6, encoding a protein or peptide from a warm-blooded animal that is a rodent or primate.

12. A nucleic acid as claimed in Claim 6 substantially homologous to the sequence of SEQ ID NO: 1.

13. An antibody specific for binding to and raised against a purified B70 antigen or a peptide of said B70 antigen as claimed in Claim 1.

14. An antibody as claimed in Claim 13, wherein:

a) said B70 antigen is a mammalian protein;
b) said antibody is raised against a peptide sequence of SEQ ID NO 2;
c) said antibody is a monoclonal antibody; or
d) said antibody is labeled.

15. An antibody as claimed in Claim 14, raised against a protein or peptide from a warm-blooded animal that is a rodent or primate.

16. A composition comprising a B70 antigen or peptide thereof as claimed in Claim 5, and a pharmaceutically acceptable carrier.

17. A kit comprising:

a) a nucleic acid encoding a B70 antigen or peptide;
b) a substantially pure B70 antigen or fragment; or
c) an antibody raised against a B70 antigen.

18. A method of modulating physiology, growth, or development of a cell, comprising contacting said cell with a non-B7/BB1 agonist or antagonist of an B70 antigen.

19. A method as claimed in Claim 18, wherein said agonist or antagonist of a B70 antigen is accompanied by an agonist or antagonist of a TcR or CD3 marker.

20. A method as claimed in Claim 18, wherein said antagonist is an antibody raised to a mammalian B70 antigen.

21. A method as claimed in Claim 18, wherein said cell is a hematopoietic cell.

22. A method as claimed in Claim 21, wherein said cell is a lymphoid cell.

23. A method as claimed in Claim 18, wherein said modulating is:

a) augmentation of a T cell response;
b) stimulation of mitogenic activity;
c) regulation of lymphokine secretion; or
d) induction of cell-mediated cytotoxicity.

24. A method as claimed in Claim 18, wherein;

a) said T cell response is selected from:
    i) immune surveillance of tumor cells; or
    ii) vaccine response;
b) said mitogenic activity is a Class I restricted or Class II restricted antigen-specific T cell clone proliferation; and
c) said lymphokine secretion includes IL-2, IFN-γ, TNF-α, lymphotoxin, GM-CSF, or IL-3.

25. A method as claimed in Claim 18, wherein said modulating is suppressing an immune response.

26. A method as claimed in Claim 18, wherein said immune response is an autoimmune response or a reaction against alloantigens.

27. A method for screening for a non-CTLA-4/CD28 B70 specific binding composition, comprising the steps of:

a) contacting a B70 antigen with a series of candidate compounds; and
b) selecting candidate compounds which bind to said B70 antigen.

Fig. 1A

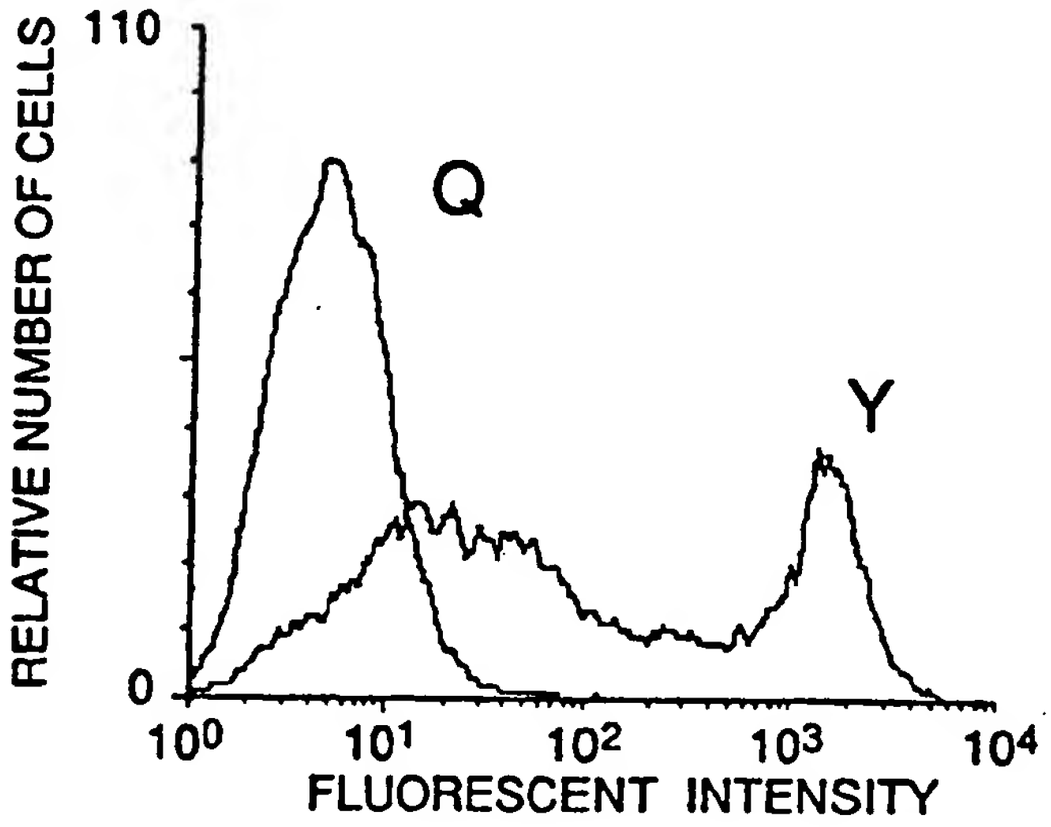

Fig. 1B

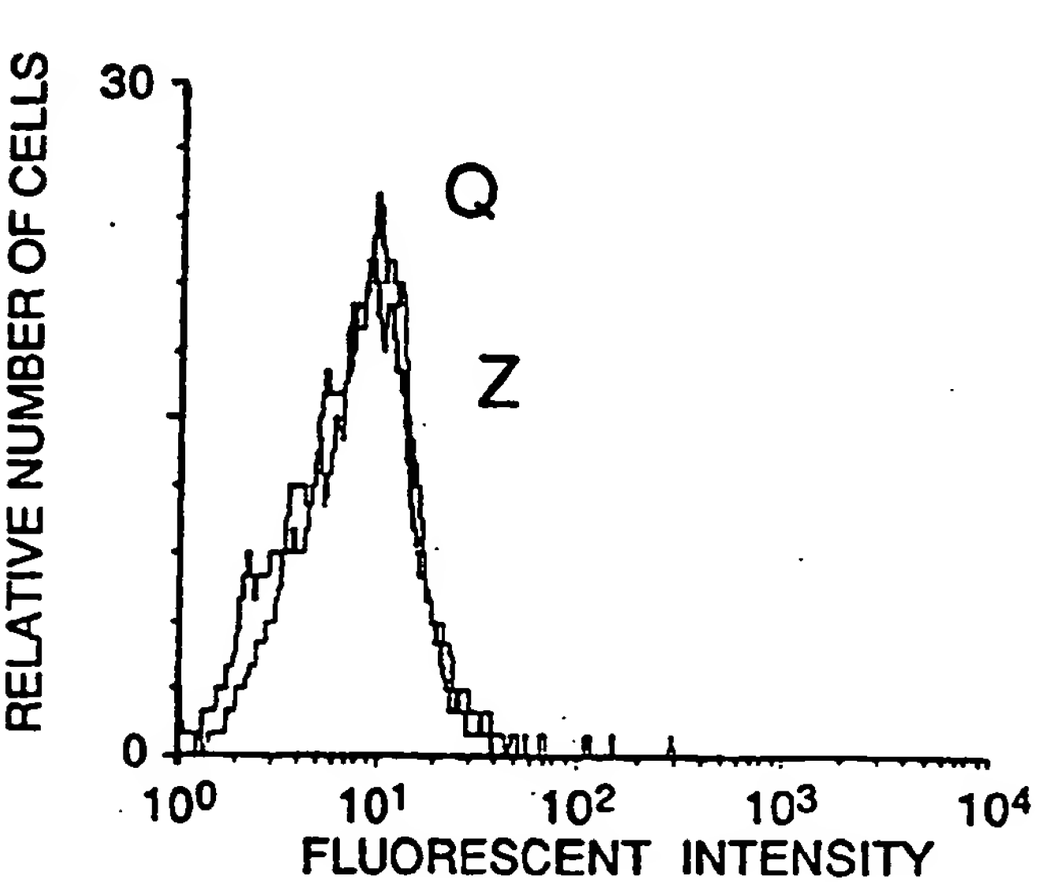

Fig. 1C

INTERNATIONAL SEARCH REPORT

Internat d Application No
PCT/US 94/09642

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K14/705 C07K16/28 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGY., vol.149, no.4, 15 August 1992, BALTIMORE US pages 1115 - 1123 AZUMA, M. ET AL.; 'Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human natural killer leukemia cell line' see the whole document ---	1-27
A	WO,A,93 00431 (BRISTOL MYERS SQUIBB) 7 January 1993 see the whole document ---	1-27
A	WO,A,92 15671 (CYTOMED, INC.; US) 17 September 1992 see the whole document ---	1-27

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 1994	21. 12. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 94/09642

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NATURE., vol.366, 4 November 1993, LONDON GB pages 76 - 79 AZUMA, M. ET AL.; 'B70 antigen is a second ligand for CTLA-4 and CD28' see the whole document ---	1-27
P,X	SCIENCE, vol.262, 5 November 1993, LANCASTER, PA FREEMAN, G.J. ET AL.; 'Cloning of B7-2 : a CTLA-4 counter receptor that costimulates human T cell proliferation."' see the whole document ---	1-27
P,X	IMMUNOLOGY TODAY, vol.15, no.7, 1 July 1994, CAMBRIDGE GB pages 321 - 332 JUNE, C.H. ET AL.; 'The B7 and CD28 receptor families.' see the whole document -----	1-27

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US94/09642

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 18-26 are directed to a method of treatment of the human/animal body as far as applied in vivo, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Interna...l Application No
PCT/US 94/09642

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9300431	07-01-93	AU-A-	2240092	25-01-93
		CA-A-	2110518	07-01-93
		EP-A-	0606217	20-07-94
		JP-T-	6508989	13-10-94
		NO-A-	934801	21-02-94
		PT-A-	100637	31-05-94
WO-A-9215671	17-09-92	EP-A-	0575537	29-12-93
		JP-T-	6505396	23-06-94